Plastin polymorphisms predict gender- and stage-specific colon cancer recurrence after adjuvant chemotherapy

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Abstract: Tumor recurrence after curative resection remains a major problem in patients with locally advanced colorectal cancer treated with adjuvant chemotherapy. Genetic single-nucleotide polymorphisms (SNP) may serve as useful molecular markers to predict clinical outcomes in these patients and identify targets for future drug development. Recent in vitro and in vivo studies have demonstrated that the plastin genes PLS3 and LCP1 are overexpressed in colon cancer cells and play an important role in tumor cell invasion, adhesion, and migration. Hence, we hypothesized that functional genetic variations of plastin may have direct effects on the progression and prognosis of locally advanced colorectal cancer. We tested whether functional tagging polymorphisms of PLS3 and LCP1 predict time to tumor recurrence (TTR) in 732 patients (training set, 234; validation set, 498) with stage II/III colorectal cancer. The PLS3 rs11342 and LCP1 rs4941543 polymorphisms were associated with a significantly increased risk for recurrence in the training set. PLS3 rs6643869 showed a consistent association with TTR in the training and validation set, when stratified by gender and tumor location. Female patients with the PLS3 rs6643869 AA genotype had the shortest median TTR compared with those with any G allele in the training set [1.7 vs. 9.4 years; HR, 2.84; 95% confidence interval (CI), 1.32–6.1; P = 0.005] and validation set (3.3 vs. 13.7 years; HR, 2.07; 95% CI, 1.09–3.91; P = 0.021). Our findings suggest that several SNPs of the PLS3 and LCP1 genes could serve as gender- and/or stage-specific molecular predictors of tumor recurrence in stage II/III patients with colorectal cancer as well as potential therapeutic targets.

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Abstract

Tumor recurrence after curative resection remains a major problem in patients with locally advanced colorectal cancer (CRC) treated with adjuvant chemotherapy. Genetic single nucleotide polymorphisms (SNPs) may serve as useful molecular markers to predict clinical outcomes in these patients and identify targets for future drug development. Recent *in vitro* and *in vivo* studies have demonstrated that the plastin genes, PLS3 and LCP1, are overexpressed in colon cancer cells and play an important role in tumor cell invasion, adhesion and migration. Hence, we hypothesized that functional genetic variations of plastin may direct effects on the progression and prognosis of locally advanced CRC. We tested whether functional tagging polymorphisms of PLS3 and LCP1 predict time to tumor recurrence (TTR) in 732 patients (training set: 234; validation set: 498) with stage II/III CRC. The PLS3 rs11342 and LCP1 rs4941543 polymorphisms were associated with a significantly increased risk for recurrence in the training set. PLS3 rs6643869 showed a consistent association with TTR in the training and validation set, when stratified by gender and tumor location. Female patients with the PLS3 rs6643869 AA genotype had the shortest median TTR compared to those with any G allele in the training set [1.7 vs. 9.4 years; HR 2.84(95% CI=1.32-6.1); p=0.005] and validation set [3.3 vs. 13.7 years; HR 2.07 (95%CI=1.09-3.91); p=0.021]. Our findings suggest that several SNPs of the PLS3 and LCP1 genes could serve as gender and/or stage-specific molecular predictors of tumor recurrence in stage II/III CRC patients as well as potential therapeutic targets.

Introduction

Colorectal cancer (CRC) is the leading cause of death from gastrointestinal malignancy, with approximately 50,830 deaths expected in 2013 (1). Surgical resection affords the only chance for cure, and adjuvant chemotherapy is recommended for high-risk stage II and all stage III tumors as it reduces risk of recurrence and prolongs disease-free survival (2, 3). However, even with adjuvant chemotherapy, 20-30% of high-risk stage II and 50-65% of stage III patients relapse within 5 years. Disease recurrence and survival are related to tumor histology, grade and stage. Such conventional clinicopathological staging has served as the standard measure of prognosis for many years. We have yet to identify reliable molecular markers to predict tumor recurrence in patients with stage II-III disease who have received adjuvant chemotherapy.

While a number of genes have been implicated in colorectal cancer metastasis, the precise mechanisms directing the migration and invasion of tumor cells into specific organs remain elusive. Modulation of the actin cytoskeleton is critical for tumor cell migration and invasion (4), and changes in the actin cytoskeleton are accomplished by a variety of actin-binding proteins, such as plastins (4, 5). Moreover, increasing evidence suggests that overexpression of plastins in cultured epithelial cells can increase cell motility (6). Therefore, plastins may not only be a potential biomarker but also serve as a valuable target for inhibiting tumor cell invasion and metastasis.

The human plastin family is comprised of three protein isoforms: T-plastin (PLS3), L-plastin (LCP1) and I-plastin. I-plastin is specifically expressed in the small intestine and kidney. The cell-type-specific expression of the other isoforms (PLS3 and

LCP1) is regulated under physiological conditions. However, ectopic expression of plastins in malignant cells has been observed in many studies (7, 8). PLS3 and LCP1, which have 80% amino acid homology, also share the unique property of cross-linking actin filaments into tight bundles. Although PLS3 and LCP1 are related in structure and function, they are encoded by two distinct genes located on chromosomes X and 13, respectively (9), and possess other unique features. Specifically, PLS3 is expressed in a wide variety of cells except for hematopoietic cells. Most neoplastic cells derived from solid tumors also express PLS3. Conversely, LCP1 is expressed in most human cancer and hematopoietic cell lines (7). PLS3 up-regulation by the lamina A filament protein has been correlated with increased invasiveness in CRC (10), and LCP1 overexpression plays a significant role in proliferation and invasion in colon cancer cell lines (11). Furthermore, LCP1 has been proposed to mediate cell adhesion and motility by binding actin (9, 12). However, little is known about the role of PLS3 and LCP1 in colorectal tumor metastasis.

Previously, data from our group and others have shown that certain genetic polymorphisms may predict clinical outcomes in colon cancer patients treated with adjuvant chemotherapy (13, 14). These common single nucleotide variations may alter gene transcription, translation or splicing, thereby causing different tumor recurrence and chemoresistance patterns. Since plastin genes play an important role in tumor cell invasion, adhesion and migration, functional variations in these genes may translate into direct effects on prognosis in patients with locally advanced colon cancer. To test this hypothesis, we performed a comprehensive study using both a training and validation set to evaluate associations between 6 functional PLS3 (4) and LCP1 (2) polymorphisms

with clinical outcomes, including TTR, in colon cancer patients treated with adjuvant chemotherapy. Lastly, we explored whether PLS3 and LCP1 polymorphisms demonstrated gender or stage-specific differences and if such differences correlated with clinical outcomes.

Materials and Methods

Eligible Patients

A total of 234 patients with stage III and high-risk stage II colon cancer were included in this study as the training set (USC cohort) (Table 1). High risk stage II disease was defined by the presence of any of the following: T4 tumor, positive surgical margins, grade 3 or 4 histology in the absence of MSI-high status, perforation, obstruction, lymphovascular or perineural invasion, or less than 12 lymph nodes sampled at time of surgical resection (15). All patients were treated with 5-FU-based adjuvant chemotherapy at the Norris Comprehensive Cancer Center/University of Southern California (NCCC/USC) or the Los Angeles County/USC-Medical Center (LAC/USCMC) from 1987 to 2007. For the validation set (Table 1), a total of 498 patients with histologically confirmed stage II (both high risk and low risk tumors) and stage III colon cancer treated at the Division of Clinical Oncology, Department of Medicine, Medical University of Graz (MUG) from 2000 to 2009 were included. All patients were treated with a 5-fluorouracil (5-FU)-based regimen. All patients were included in the colon cancer surveillance program of NCCC/USC, LAC/USCMC, or MUG, providing the following information: history and physical examination and carcinoembryonic antigen (CEA) levels every 3 months for 3 years and every 6 months at

years 4 and 5 after surgery; colonoscopy at year 1 and thereafter every 3–5 years; and computed tomography scans of chest and abdomen every 6 months for the first 3 years. Patient data were collected retrospectively through chart review. Whole blood was collected at the time of diagnosis and stored at −80□°C. The study was approved by the Institutional Review Boards of MUG and USC, and all study participants signed informed consent for the analysis of molecular correlates.

Candidate Polymorphisms

Stringent pre-defined criteria were used to select candidate single nucleotide polymorphisms (SNPs) and included: (a) polymorphisms which could alter gene function in a biologically relevant manner (as predicted by the Functional-Single-Nucleotide-Polymorphism (F-SNP) database) (16, 17); (b) a minor allele frequency ≥10% in Caucasians (with relative allelic frequencies of polymorphisms in different ethnicities obtained from the genetics section in the Ensembl Genome Browser: http://uswest.ensembl.org/index.html). This study focused on testing the potentially functional genetic variants located at the 5'UTR, 3'UTR, or coding region, as well as tagging SNPs. (Supplementary Table 1).

Genotyping

Genomic DNA was extracted from peripheral blood using the QIAmp-kit (Qiagen) and according to the manufacturer's instructions (www.qiagen.com). For the USC cohort, the candidate polymorphisms were tested using PCR-based direct DNA sequence analysis. Briefly, forward and reverse primers were used for PCR amplification.

PCR fragments were sequenced on an ABI 3100A Capillary Genetic Analyzer (Applied Biosystems) and analyzed in either sense or antisense directions for the presence of the polymorphism. DNA sequence analyses were performed using the ABI Sequencing Scanner v1.0 (Applied Biosystems). The investigator analyzing the germline polymorphisms was blinded to the clinical dataset. For the MUG cohort, genotypes were centrally determined by a 5'-exonuclease assay (TaqMan) at the Medical University of Graz. Primer and probe sets were designed and manufactured using Applied Biosystems 'Assay-by-Design' custom service (Applera, Austria). General TaqMan reaction conditions were according to the manufacturer of the assays. End-point fluorescence was measured in a Lambda Fluoro 320 plus plate reader (MWG Biotech AG, Germany) using excitation/emission filters of 485/530 nm and 530/572 nm, respectively. In the plot, genotype groups were identified as separate and distinguishable clusters. To ensure methodological consistency and quality control, genotype determination was repeated in at least 96 randomly selected samples.

Statistical Analysis

The primary endpoint of this study was time to tumor recurrence (TTR), which was computed from the date of diagnosis of colon cancer to the date of the first documented tumor recurrence. If tumor recurrence was not observed, TTR was censored at the time of death or at the last follow-up. Deviation from Hardy-Weinberg equilibrium for allelic distributions of PLS3 and LCP1 SNPs were tested using a 1-degree-freedom χ^2. The relationships between PLS3 and LCP1 SNPs and baseline patient and tumor characteristics were examined using χ^2 or Fisher's exact test whenever appropriate. The

associations between PLS3 and LCP1 SNPs and TTR were assessed using Kaplan-Meier curves and log-rank tests in the univariate analysis, and Cox regression models adjusting for stage and type of adjuvant therapy, stratified by race in the multivariate analysis. As the PLS3 gene is located on X chromosome, the analyses for all PLS3 SNPs were conducted separately by gender. We tested the hypothesis that plastin polymorphisms predict time to recurrence in patients with stage II or III colon cancer in our USC institutional cohort first. These findings were then validated in an independent cohort. Concordance probability estimates (CPE) for the Cox proportional hazards model were calculated to evaluate the incremental contribution in discrimination provided by the PLS3 and LCP1 SNPs over the baseline prognostic factors in both cohorts (18).

SAS 9.3 (SAS Institute, Cary, NC, USA) and R package 'CPE' were used to perform all the analyses. All tests were 2-sided at a significance level of 0.05.

Results

The baseline characteristics of the training set and validation set patients included in this analysis are summarized in Table 1. All genotype frequencies for plastin polymorphisms analyzed were within Hardy-Weinberg equilibrium (HWE) ($p>0.05$).

Training Set

A total of 234 USC patients with stage II and III CRC were included in this analysis as the training set (14). All patients were diagnosed with stage III and high risk stage II CRC during the years of 1987 and 2007. The group consisted of 107 women (46%) and 127 men (54%), with a median age of 59 years (range 22–87 years). The

median follow-up was 4.4 years (range 0.4-16.8 years). Of the 234 CRC patients, ninety (38.5%) patients showed tumor recurrence, with a stage III and high-risk stage II dependent 3-year recurrence probability of 0.45 ± 0.047 and 0.21 ± 0.043, respectively. The median TTR was 7.1 years (95% CI 4.9-16.8+ years), and forty-four (19%) of the 234 patients died. The median OS for this cohort has not yet been reached. Patients with stage III disease were more likely to present with recurrence, compared with patients with high risk stage II disease (log-rank $p<0.001$). We did not observe any significant associations between other demographic and clinicopathologic variables and TTR. In the USC cohort, plastin polymorphisms were not significantly associated with age, clinical (type of chemotherapy administered) or pathological characteristics (tumor stage, grade or lymph node status) ($p>0.05$).

Validation Set

In order to validate our results, we performed an analysis of the polymorphisms, PLS3 rs6643869 and LCP1 rs4941543, in an independent Austrian cohort of 498 patients with stage II and III CRC. Baseline patient characteristics, tumor biological factors and therapeutic modalities are shown in Table 1. The median age at time of diagnosis was 66 years (range 27-95 years). The median follow-up time was 64 months (range 1-186 months). The median OS of this cohort was 15.3 years (95% CI 9.7-15.5+ years).

Single Nucleotide Polymorphism (SNP) Analysis

Training Set

Genotyping of PLS3 rs6643869 and LCP1 rs4941543 was successful in 180 (77%) of the 234 patients. In the remaining 54 (23%) cases, there was limited quantity

and/or quality of extracted genomic DNA. As the PLS3 gene is located on the X chromosome, data were analyzed separately for men and women for the PLS3 polymorphism. In univariate analysis, PLS3 rs6643869 and LCP1 rs4941543 were significantly associated with time to tumor recurrence (TTR). Female patients with the PLS3 rs6643869 A/A genotype had a shorter median TTR of 1.7 years (95% CI 0.7-5.9+ years) compared to those with the A/G or G/G genotypes, who had a median TTR of 9.4 years (95% CI 5.4-12.2+ years) (HR=2.84, 95% CI 1.32-6.10; p=0.005, log-rank test) (Figure 1, top panel; Table 2). We further analyzed female patients with PLS3 rs6643869 by tumor location. Both sides (right and left) have similar results (Supplementary Figure 1). All patients with the LCP1 rs4941543 C/C genotype had a longer median TTR of 10.7 years (95% CI 5.7-16.8+ years) compared to those with the C/T and T/T genotypes, who had a median TTR of 4.9 years (95% CI 2.0-9.0+ years) (HR=1.83, 95% CI 1.01-3.30; p=0.040, log-rank test) (Figure 1, bottom panel; Supplementary Table 2). In addition, male patients with the LCP1 rs11342 T allele had a longer median TTR of 7.1 years (95% CI 4.9-11.4+ years), relative to men with the G/G genotype who had a median TTR 2.3 years, (95% CI 1.5-6.8+ years) (HR=0.51, 95% CI 0.25-1.04; p=0.042) in univariate analysis (Supplementary Figure 2, Table 2).

The multivariate model for the USC cohort was adjusted for stage and type of chemotherapy as covariates and stratified by race and gender. The A/A genotype (n=14) of PLS3 rs6643869 demonstrated a non-significant trend towards decreased TTR (HR=2.26, 95% CI 0.97-5.31; adjusted p=0.06) compared with patients with the A/G or G/G genotypes (n = 68) in female patients (Table 2). CPE was 0.728 (standard error (SE): 0.039) and 0.741 (SE: 0.037) for the multivariate model including baseline patient

prognostic factors only (baseline model), and adding PLS3 rs6643869 into the model among female patients, respectively. LCP1 rs4941543 was not associated with TTR in multivariate analysis (adjusted p=0.11) in any population. However, amongst female patients, those with the C/C genotype had a lower risk of recurrence than carriers of the T allele (HR=2.95, 95% CI 1.10-7.90; adjusted p=0.031). CPE was 0.769 (SE: 0.034) for LCP1 rs4941543-added multivariate model and 0.744 (SE: 0.034) for the baseline model. There were no statistically significant differences between the PLS3 rs6643869, LCP1 rs4941543 and LCP1 rs11432 polymorphisms and TTR in male patients (Supplementary Figure 3; Table 2). These data suggest that the PLS3 rs6643869 and LCP1 rs4941543 polymorphisms may serve as gender-based prognostic factors. We did not observe statistically significant associations between the other tested genes with regards to time to disease relapse (Supplementary Table 3).

Validation Set

In the overall population analysis, the PLS3 rs6643869 and LCP1 rs4941543 polymorphisms were not associated with TTR in the validation set. However, when patients were stratified by gender, tumor location and stage, the PLS3 rs6643869 and LCP1 rs4941543 polymorphisms demonstrated the following significant relationships:

1) In right-sided CRC, female patients with any G allele of PLS3 rs6643869 had an increased TTR (13.7+ years) compared to the G/A and A/A genotypes (3.3 years) (Figure 2, top panel) in both univariate (HR=2.07, 95% CI 1.09-3.91; p=0.021) and multivariate analyses (HR=2.41, 95% CI 1.22-4.76; p=0.011) (Table 3, top right panel). CPE was 0.639 (SE: 0.037) and 0.672 (SE: 0.034) for the baseline multivariate model and PLS3

rs6643869-added model, respectively. In men, PLS3 rs6643869 was not significantly associated with TTR (Table 3, bottom left panel). These data suggest that the PLS3 rs6643869 polymorphism is a positive prognostic indicator whose influence depends upon both gender and tumor location.

2) Women with the LCP1 rs4941543 C/C genotype had a shorter TTR (10 years, 95% CI 6.9-14.1+ years) compared to those with any T allele in univariate analysis (TTR 12.2+ years; HR=0.53, 95% CI 0.29-0.97; p=0.035) (Figure 2, bottom panel) and remained significantly associated in multivariate analysis (HR=0.53, 95% CI 0.29-0.97; p=0.039) (Table 3, bottom panel). CPE was 0.650 (SE: 0.029) and 0.667 (SE: 0.028) for the baseline multivariate and LCP1 rs4941543-added model, respectively. These data are in contradiction to that found in our USC training cohort, which showed the C/C genotype to correlate with decreased risk of recurrence.

3) In stage III CRC, female patients with the LCP1 rs4941543 C/C genotype had a significantly decreased TTR (4.1 years) than carriers of the T allele in univariate analysis (10.6 years) (HR=0.47, 95% CI 0.23-0.96, p=0.031), with a similar but non-significant trend in multivariate analysis (HR=0.49, 95% CI 0.24-1.01; p=0.052) (Table 4). These data support the role of LCP1 rs4941543 as a prognosticator based on gender and tumor stage.

Discussion

In this study, we demonstrated that germline polymorphisms within the PLS3 and LCP1 genes are significantly associated with time to tumor recurrence in patients with locally advanced colon cancer treated with adjuvant chemotherapy. Moreover, when stratified by gender, the PLS3 rs6643869 and LCP1 rs4941543 polymorphisms correlate

with TTR in women. The correlation between clinical outcomes and PLS3 rs6643869 was validated in an independent Austrian cohort. To our knowledge, this is the first report suggesting a relationship between PLS3 and LCP1 polymorphisms and clinical outcomes in stage II/III colon cancer patients that is dependent upon gender, tumor location, and stage. These findings suggest that plastins may serve as a useful prognostic biomarker and warrant further investigation.

Our study identified several SNPs in the PLS3 and LCP1 genes that may play a significant role in predicting outcomes of stage II/III colon cancer patients. Patients with the PLS3 rs6643869 polymorphism A/A genotype in the intron region of the PLS3 gene had a significantly higher risk of tumor recurrence compared with patients carrying at least one G allele in both the training and validation sets. Patients carrying the LCP1 rs4941543 polymorphism C/C genotype in the exon region of the LCP1 gene had a significantly lower risk of tumor recurrence compared with patients possessing either C/T or T/T in our training set. However, our validation set showed an opposite relationship between the C/C genotype and outcomes. This discrepancy could be attributed to two reasons. First, differences in ethnicity between the two populations may translate into contrasting pharmacogenomic interactions or activation of alternate pathways which affect tumor cell biology and behavior. Secondly, whereas the training set included only high risk stage II patients, the validation cohort included both high and low risk stage II patients (as we could not discern high or low risk status from the available clinical data). Low risk stage II patients may have a longer TTR and are more likely to live long enough to develop a recurrence.

The molecular mechanisms by which plastin polymorphisms affect tumor behavior and recurrence are under investigation. Modulations of transcription, interconnected stem cell pathways, and treatment-related factors have all been implicated. With regards to transcription, we know that the PLS3 rs6643869 polymorphism is located at intron 2 of PLS3 gene, and the SNP functional prediction tool (F-SNP) has shown that a single nucleotide change from A to G may alter transcription factor binding of this gene. For example, PLS3 rs6643869 containing the G allele but not the A allele binds two transcription factors, Tst-1 and HSF, which may affect subsequent transcriptional activity in a process known as intron-mediated enhancement. With regards to the LCP1 rs4941543 polymorphism located at exon 18, a missense mutation from C to T renders an amino acid change from lysine (K) to glutamine (E), which may lead to potential functional alterations. Moreover, our findings suggest that PLS3 rs6643869 and LCP1 rs4941543 are gender-specific markers, which may imply further hormonal interactions. The fact that PLS3 maps to X.q23 may partly account for its gender-specific effect, but additional studies are needed to better elucidate the mechanisms underlying these associations.

Once deranged transcription has occurred, plastins may then mediate colon cancer metastasis through two distinct mechanisms. The first involves the Epithelial-Mesenchymal Transition (EMT) which is critical for cancer development and metastasis (19, 20). A key effector of this transition is E-cadherin, and E-cadherin expression levels have reliably demonstrated an inverse correlation with tumor invasiveness. It follows that loss of E-cadherin expression occurs at sites of EMT during tumor progression (21, 22). Therefore, one possibility is that overexpressed PLS3 and LCP1 in colon cancer cells

lead to loss of E-cadherin (10, 11) and thereby promotes β-catenin release through the Wnt-signaling pathway (23, 24), facilitating passage through the EMT. Alternatively, plastin overexpression may enhance cell motility by modifying the actin cytoskeleton and, in doing so, increase metastatic potential.

Studies using CRC cell lines have shown a promising link between PLS3, the epithelial mesenchymal transition, and tumor metastasis. Willis and colleagues (10) showed that lamina A, a putative colon stem cell biomarker, can induce PLS3 upregulation, which in turn leads to E-cadherin downregulation and increased tumor invasiveness. Furthermore, a recent study led by Yokobori reported that PLS3 itself may be a novel circulating tumor cell (CTC) marker expressed during the epithelial mesenchymal transition (EMT), and the detection of PLS3-positive CTCs was found to be an independent prognostic factor in metastatic CRC patients (25). Our study showed that PLS3 plasma concentration was significantly higher in CRC patients compared to control patients with no evident disease (NED) (Supplementary Figure 4), which is consistent with previous studies, and suggests that PLS3 expression may serve as a surrogate for tumor invasiveness in stage II/III CRC.

Other studies have similarly demonstrated a connection between LCP1, E-cadherin mediated tumor spread, and poor prognosis in colon cancer. Elevated LCP1 levels in cancer cell lines and solid tumors have been correlated with increased migration, invasion and metastatic potential (12, 26, 27). In CRC, several independent studies have confirmed that L-plastin protein overexpression is also a marker for aggressive disease and poor prognosis (28, 29). Foran *et al*. (11) showed that CRC cell lines overexpressing LCP1 acquire invasiveness through E-cadherin downregulation, which induces

dedifferentiation, invasion and metastasis. Furthermore, others (29, 30) have shown that LCP1 correlates with tumor protein expression and CRC progression using immunohistochemical analysis and ELISA, which is consistent with our results in the USC cohort. Our data showed that LCP1 plasma concentration was much higher in CRC patients than NED control patients. Moreover, LCP1 was higher in stage III than stage II patients, suggesting that LCP1 expression may be related to lymph node metastasis (Supplementary Figure 3).

In addition to affecting the intrinsic invasiveness of tumor cells, PLS3 has been implicated in modulating chemotherapeutic efficacy. Specifically, enhanced PLS3 expression has been associated with both chemotherapy and radiation resistance in cancer cells (31, 32). Hisano *et al.* reported that PLS3 downregulation led to increased sensitivity to cisplatin in platinum-resistant cell lines. In our future studies, we will focus on the correlation between plastin expression levels and the presence of LCP1 or PLS3 polymorphisms. The PLS3 rs6643869 and LCP1 rs4941543 polymorphisms, in particular, may alter gene function/expression, thereby increasing PLS3 and LCP1 levels and promoting Wnt signaling through the E-cadherin/ß-catenin complex, ultimately resulting in CRC recurrence and progression.

In summary, this study provides the first evidence that germline genetic variants of the PLS3 and LCP1 genes predict tumor recurrence and time to disease relapse in a gender-and stage-specific manner. The PLS3 rs6643869 SNP retained a significant correlation with outcomes after adjusting for multiple variables and has now been confirmed in a validated cohort. Taken together, our data strengthen the role of plastin

proteins in locally advanced CRC and may aid in the selection of patients who would benefit from plastin-targeted treatments in the future.

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Table 1. Colorectal cancer baseline patient characteristics

	Training set (USC cohort)		Validation set (Austrian cohort)		*P* value*
	n	%	n	%	
Age, years					
<55	86	36.75	87	17.47	
55-64	82	35.04	138	27.71	
≥65	66	28.21	273	54.82	<0.001
Sex					
Female	107	45.72	230	46.18	
Male	127	54.28	268	53.82	0.91
T					
T1	2	0.85	10	2.01	
T2	14	5.98	22	4.42	
T3	187	79.91	358	71.89	<0.001
T4	27	11.54	108	21.69	
Tx	4	1.72	0		
N					
Negative	105	44.87	192	38.55	
N1	72	30.77	195	39.16	
N2	57	24.36	111	22.29	0.085
Grade					
Well	11	2.18	25	5.02	
Moderate	151	64.53	326	65.46	
Poor/undifferentiated	54	23.08	145	29.12	0.51
Missing	18	10.21	2	0.40	
Stage					
II	105	44.87	192	38.55	
III	129	55.13	306	61.45	0.10
N of resected lymph nodes					
≤12	70	29.91	72	14.46	
>12	145	61.97	426	85.54	<0.001
Missing	19	8.12	0	0	
Tumor side					
Left	110	47.01	188	37.75	
Right	115	49.15	310	62.25	0.005
Left and right	4	1.71	0		
Missing	5	2.13	0		
Adjuvant treatment					
5-FU	151	64.53	171	34.41	
5-FU/LV/Oxaliplatin	60	25.64	326	65.59	<0.001
5-FU/LV/Irinotecan	23	9.83	0	0	
Ethnicity					
Asian	34	14.53	0	0	
African American	15	6.41	0	0	
Caucasian	123	52.56	498	100	<0.001
Hispanic	62	26.5	0	0	

*Based on χ^2 test

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Table 2. PLS3 and LCP1 polymorphisms and outcome in patients with stage II or III colon cancer by sex (Training set)

	Males						Females				
	N	Median TTR, y (95% CI)	Prob ± SE* of 3-y recurrence	HR (95% CI) Univariate	HR (95% CI) Multivariate		*N*	Median TTR, y (95% CI)	Prob ± SE* of 3-y recurrence	HR (95% CI) Univariate	HR (95% CI) Multivariate
PLS3 rs6643869											
G/G	66	7.1 (3.5, 11.4+)	0.33 ± 0.07	1 (Reference)	1 (Reference)		35	12.2+ (4.9, 12.2+)	0.29 ± 0.08	1 (Reference)	1 (Reference)
G/A	27	5.2 (1.7, 10.7+)	0.36 ± 0.10	1.38 (0.69, 2.75)	1.48 (0.71, 3.08)		33	9.4 (5.4, 9.4+)	0.20 ± 0.08	0.89 (0.37, 2.14)	0.75 (0.30, 1.92)
A/A				0.35	0.30		14	1.7 (0.7, 5.9+)	0.67 ± 0.13	2.69 (1.14, 6.35)	1.96 (0.75, 5.13)
P†										0.018	0.15
G/G or G/A							68	9.4 (5.4, 12.2+)	0.25 ± 0.06	1 (Reference)	1 (Reference)
A/A							14	1.7 (0.7, 5.9+)	0.67 ± 0.13	2.84 (1.32, 6.10)	2.26 (0.97, 5.31)
P†										**0.005**	0.060
LCP rs11342											
G/G	33	2.3 (1.5, 6.8+)	0.52 ± 0.11	1 (Reference)	1 (Reference)		23	10.3+	0.23 ± 0.09	1 (Reference)	1 (Reference)
G/T	42	10.7 (5.2, 11.4+)	0.17 ± 0.06	0.39 (0.17, 0.89)	0.36 (0.14, 0.92)		44	5.4 (1.8, 9.4+)	0.40 ± 0.08	2.27 (0.86, 6.03)	1.53 (0.50, 4.63)
T/T	21	3.9 (1.4, 10.7+)	0.41 ± 0.11	0.78 (0.34, 1.83)	0.71 (0.29, 1.73)		17	12.2+ (2.8, 12.2+)	0.21 ± 0.11	1.00 (0.27, 3.71)	0.68 (0.16, 2.85)
P†				0.037	0.096					0.10	0.31
G/T, T/T	63	7.1 (4.9, 11.4+)	0.25 ± 0.06	0.51 (0.25, 1.04)	0.50 (0.23, 1.10)		61	5.7 (2.8, 16.8+)	0.35 ± 0.07	1.89 (0.72, 4.93)	1.29 (0.43, 3.85)
P†				**0.042**	0.085					0.19	0.65
LCP rs4941543											
C/C	75	10.7 (5.2, 11.4+)	0.30 ± 0.06	1 (Reference)	1 (Reference)		66	9.4 (5.4, 16.8+)	0.27 ± 0.06	1 (Reference)	1 (Reference)
C/T‡	14	4.9 (1.0, 7.1+)	0.35 ± 0.13	1.75 (0.77, 3.97)	1.60 (0.64, 3.97)		15	2.4 (0.7, 9.0+)	0.52 ± 0.14	1.91 (0.81, 4.52)	2.95 (1.10, 7.90)
T/T‡	1						0				
P†				0.17	0.32					0.13	**0.031**

* Greenwood SE.

\+ Estimates were not reached.

†Based on log-rank test in the univariate analysis and based on Wald test within multivariate Cox proportional hazards model adjusting for stage and type of adjuvant therapy and stratified by race.

‡In the dominant model.

Table 3. Associations between Plastin polymorphisms and disease-free survival in Austrian female patients with stage II or III colon cancer by tumor site and gender (validation set)

	Left Colon						Right Colon				
	N	Median TTR, y (95% CI)	Probability ± SE* of 3-year recurrence	HR (95% CI) in univariate analysis	HR (95% CI) in multivariate analysis		*N*	Median TTR, y (95% CI)	Probability ± SE* of 3-year recurrence	HR (95% CI) in univariate analysis	HR (95% CI) in multivariate analysis
PLS3 rs6643869											
G/G	27	10.8+ (0.9, 10.8+)	0.36 ± 0.10	1 (Reference)	1 (Reference)		52	13.7+ (4.6, 13.7+)	0.25 ± 0.07	1 (Reference)	1 (Reference)
G/A	41	10.6+ (3.5, 10.6+)	0.31 ± 0.08	0.84 (0.37, 1.90)	0.67 (0.29, 1.58)		58	13.2+	0.28 ± 0.06	0.93 (0.45, 1.90)	0.99 (0.47, 2.06)
A/A	12	11.6+ (1.7, 11.6+)	0.11 ± 0.10	0.20 (0.03, 1.53)	0.23 (0.03, 1.79)		25	3.3 (1.4, 15.0+)	0.46 ± 0.10	1.98 (0.94, 4.17)	2.40 (1.09, 5.27)
P†				0.23	0.31					0.068	0.040
G	68	10.8+ (3.7, 10.8+)	0.33 ± 0.06	1 (Reference)	1 (Reference)		110	13.7+	0.26 ± 0.05	1 (Reference)	1 (Reference)
A/A	12	11.6+ (1.7, 11.6+)	0.11 ± 0.10	0.22 (0.03, 1.61)	0.29 (0.04, 2.15)		25	3.3 (1.4, 15.0+)	0.46 ± 0.10	2.07 (1.09, 3.91)	2.41 (1.22, 4.76)
P†				0.099	0.22					**0.021**	**0.011**
LCP1 rs4941543											
C/C	59	11.6+ (2.3, 11.6+)	0.37 ± 0.07	1 (Reference)	1 (Reference)		106	10.0 (6.9, 14.1+)	0.33 ± 0.05	1 (Reference)	1 (Reference)
C/T‡	23	10.6+	0.17 ± 0.08	0.47 (0.18, 1.24)	0.42 (0.16, 1.15)		31	12.2+	0.23 ± 0.08	0.58 (0.27, 1.24)	0.59 (0.27, 1.28)
T/T‡	1						2				
P†				0.11	0.092					0.15	0.18
	Male						**Female**				
	N	Median TTR, y (95% CI)	Probability ± SE* of 3-year recurrence	HR (95% CI) in univariate analysis	HR (95% CI) in multivariate analysis		*N*	Median TTR, y (95% CI)	Probability ± SE* of 3-year recurrence	HR (95% CI) in univariate analysis	HR (95% CI) in multivariate analysis
PLS3 rs6643869											
G/G	139	14.4 (14.4, 14.5+)	0.27 ± 0.04	1 (Reference)	1 (Reference)		79	13.7+ (6.9, 13.7+)	0.29 ± 0.06	1 (Reference)	1 (Reference)
G/A	100	12.0+ (4.0, 12.0+)	0.31 ± 0.05	1.36 (0.85, 2.19)	1.42 (0.88, 2.30)		99	13.2+	0.29 ± 0.05	0.91 (0.53, 1.55)	0.88 (0.51, 1.52)
A/A							37	10.0 (2.1, 15.0+)	0.36 ± 0.08	1.24 (0.65, 2.37)	1.43 (0.73, 2.80)
P†				0.19	0.15					0.61	0.33
LCP1 rs4941543											
C/C	196	14.4 (14.4, 15.0)	0.28 ± 0.03	1 (Reference)	1 (Reference)		165	10.0 (6.9, 14.1+)	0.34 ± 0.04	1 (Reference)	1 (Reference)
C/T‡	58	8.6 (3.9, 11.4+)	0.32 ± 0.07	1.16 (0.69, 1.95)	1.26 (0.74, 2.13)		54	12.2+	0.21 ± 0.06	0.53 (0.29, 0.97)	0.53 (0.29, 0.97)
T/T‡	4						3				
P†				0.58	0.39					**0.035**	**0.039**

* Greenwood SE.

†Based on log-rank test in the univariate analysis and based on Wald test within multivariate Cox proportional hazards model adjusting for age, stage, and adjuvant therapy.

+ Estimates were not reached. ‡In the dominant model.

Table 4. Associations between Plastin polymorphisms and disease-free survival in Austrian female patients with stage II or III colon cancer by stage (Validation set)

	Stage II						Stage III				
	N	Median TTR, y (95% CI)	Probability ± SE* of 3-year recurrence	HR (95% CI) in univariate analysis	HR (95% CI) in multivariate analysis		*N*	Median TTR, y (95% CI)	Probability ± SE* of 3-year recurrence	HR (95% CI) in univariate analysis	HR (95% CI) in multivariate analysis
PLS3 rs6643869											
G/G	28	12.2+ (4.6, 12.2+)	0.18 ± 0.08	1 (Reference)	1 (Reference)		51	13.7+ (2.9, 13.7+)	0.35 ± 0.07	1 (Reference)	1 (Reference)
G/A	37	13.2+	0.10 ± 0.06	0.42 (0.12, 1.49)	0.40 (0.11, 1.41)		62	10.6+ (2.3, 10.6+)	0.39 ± 0.06	1.09 (0.60, 1.99)	1.03 (0.56, 1.89)
A/A	19	10.0 (3.3, 15.0+)	0.17 ± 0.09	1.03 (0.32, 3.26)	0.90 (0.28, 2.91)		18	2.1 (1.1, 11.6+)	0.58 ± 0.13	1.52 (0.68, 3.39)	1.43 (0.63, 3.24)
P†				0.29	0.31					0.57	0.66
G	65	13.2+	0.14 ± 0.05	1 (Reference)	1 (Reference)		113	13.7+ (3.5, 13.7+)	0.37 ± 0.05	1 (Reference)	1 (Reference)
A/A	19	10.0 (3.3, 15.0+)	0.17 ± 0.09	1.59 (0.57, 4.45)	1.47 (0.52, 4.17)		18	2.1 (1.1, 11.6+)	0.58 ± 0.13	1.44 (0.70, 2.97)	1.41 (0.68, 2.93)
P†				0.36	0.47					0.31	0.36
LCP1 rs4941543											
C/C	64	14.1+ (8.5, 14.1+)	0.15 ± 0.05	1 (Reference)	1 (Reference)		101	4.1 (1.8, 13.7+)	0.46 ± 0.05	1 (Reference)	1 (Reference)
C/T‡	23	12.2+	0.15 ± 0.08	0.75 (0.24, 2.31)	0.64 (0.21, 1.98)		31	10.6+	0.25 ± 0.08	0.47 (0.23, 0.96)	0.49 (0.24, 1.01)
T/T‡	1						2				
P†				0.61	0.44					**0.031**	**0.052**

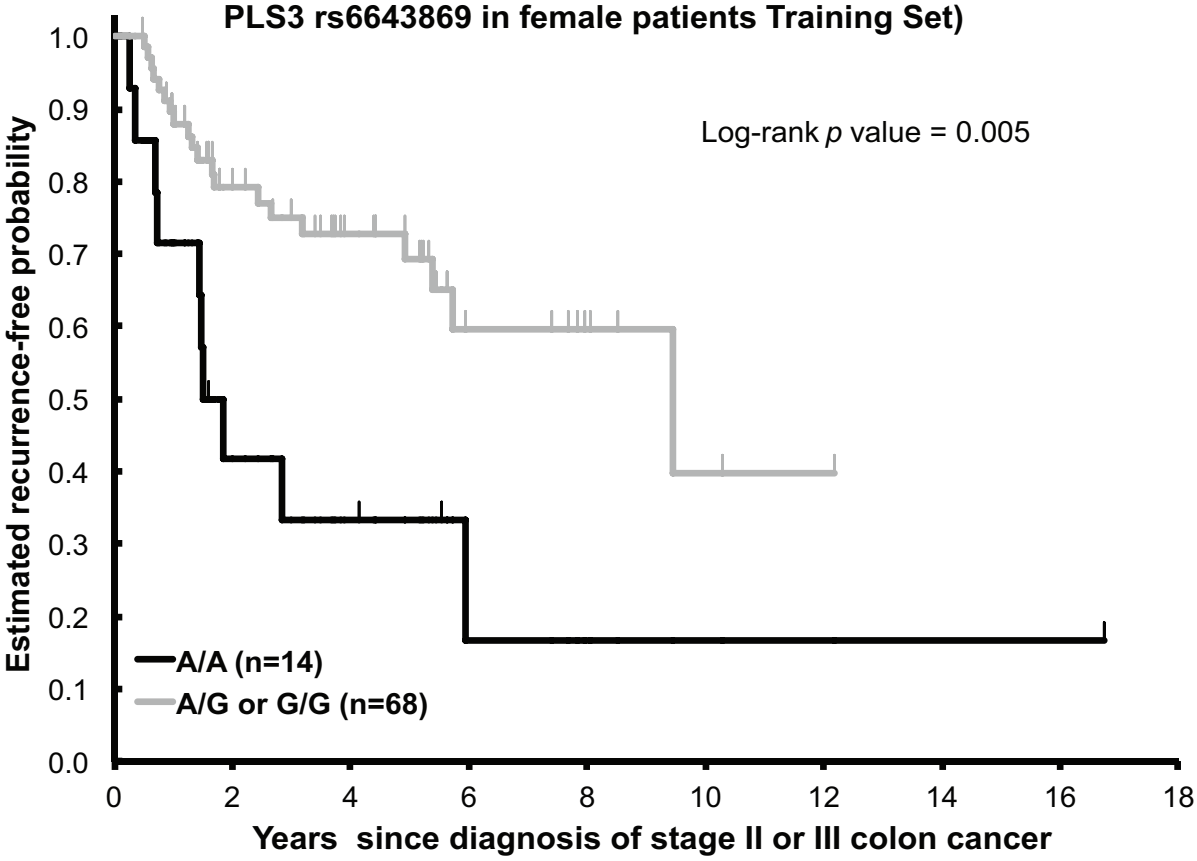
* Greenwood SE.
\+ Estimates were not reached.
†Based on log-rank test in the univariate analysis and based on Wald test within multivariate Cox proportional hazards model adjusting for age, stage, and adjuvant therapy.
‡In the dominant model.

Figure legends.

Figure 1. Time-to-tumor recurrence (TTR) by PLS3rs6643869 and LCP14941543 in USC cohort (Training set) stage II/III CRC patients treated with 5-fluorouracil (5-FU)-based chemotherapy.

Figure 2. Time-to-tumor recurrence (TTR) by PLS3rs6643869 and LCP14941543 in Austrian cohort (Validation set) stage II/III CRC patients treated with 5-fluorouracil (5-FU)-basedchemotherapy.

Figure 1.



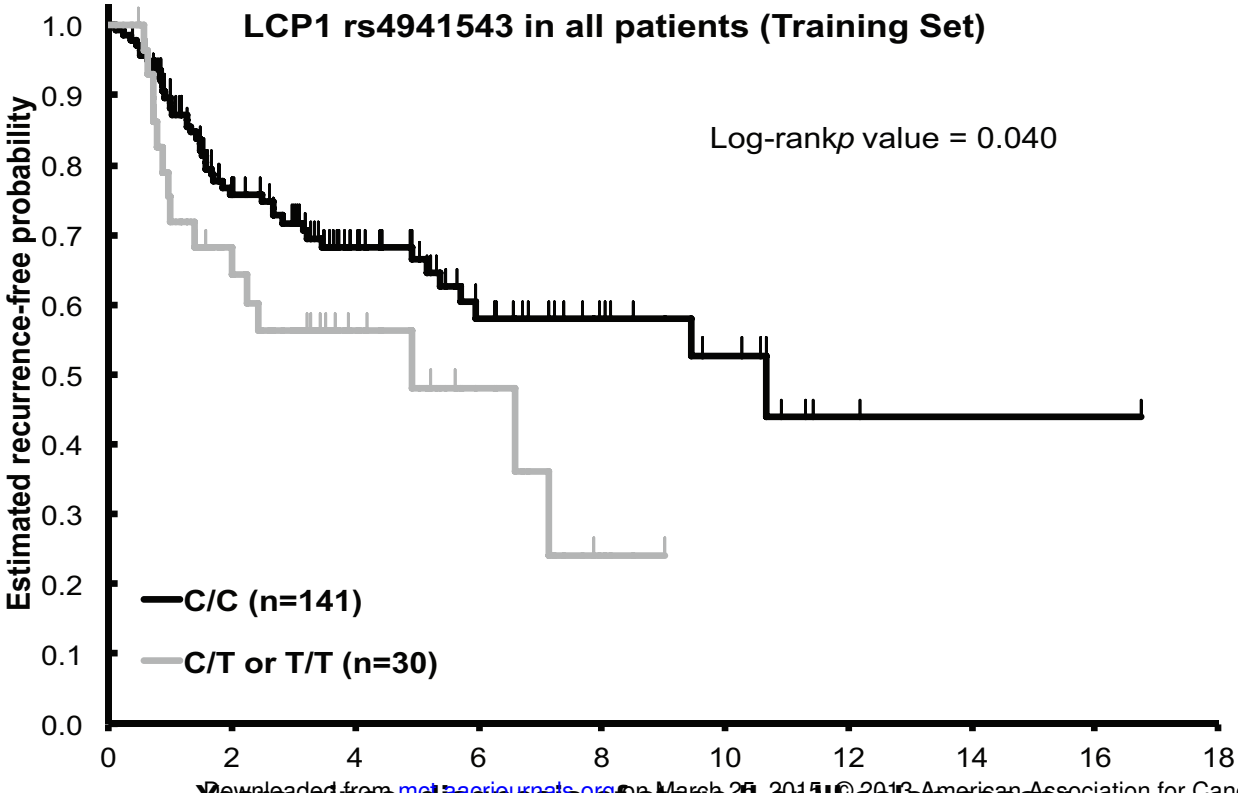
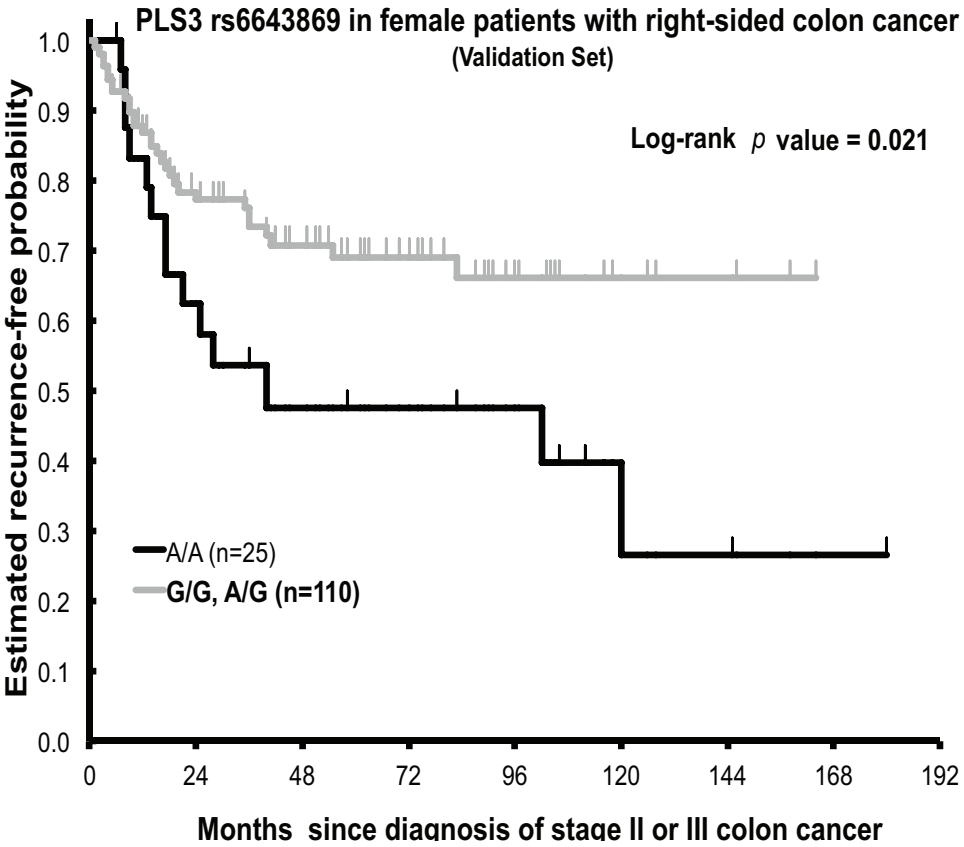
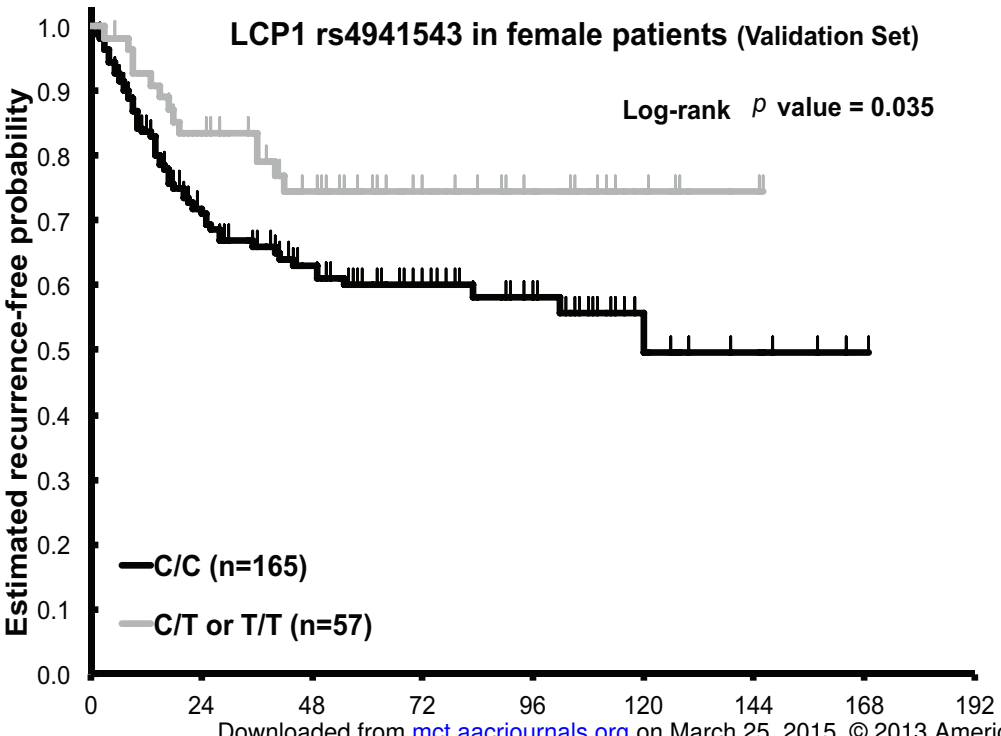


Figure 2.





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